Recombinant expression of S-layer proteins

Description

The present invention concerns processes for the recombinant production of S-layer proteins and modified S-layer proteins in gram-negative host cells.

Crystalline bacterial cell surface layers (S-layers) form the outermost cell wall component in many eubacteria and most of the archaebacteria (Sleytr et al. (1988), Crystalline Bacterial Cell Surface Layers, "Springer Verlag Berlin"; Messner and Sleytr, Adv. Microb. Physiol. 33 (1992), 213-275). Most of the presently known S-layer proteins are composed of identical proteins or glycoproteins which have apparent molecular weights in the range of 40,000 to 220,000. The components of S-layers are self-assembling and most of the lattices have an oblique (p2), quadratic (p4) or hexagonal (p6) symmetry. The functions of bacterial S-layers are still not completely understood but due to their location on the cell surface the porous crystalline S-layers probably serve mainly as protective coatings, molecular sieves or to promote cell adhesion and surface recognition.

Genetic data and sequence information are known for various S-layer genes from microorganisms. A review may be found in Peyret et al., Mol. Microbiol. 9 (1993), 97-109. Explicit reference is made to these data. The sequence of the sbsA gene coding for the S-layer protein of B.stearothermophilus PV72 and a process for cloning it are stated in Kuen et al. (Gene 145 (1994), 115-120).

B.stearothermophilus PV72 is a gram-positive bacterium which is covered with a hexagonally arranged S-layer. The main component of the S-layer is a 128 kd protein which is the most frequent protein in the cell with a proportion of about 15 % relative to the total protein components. Various strains of B.stearothermophilus have been characterized which differ with regard to the type of the S-layer lattice, the molecular weight and glycosilation of the S-layer components (Messner and Sleytr (1992), supra).

The German Patent Application P 44 25 527.6 discloses the signal peptide-coding section of the S-layer gene from B.stearothermophilus and the amino acid sequence derived therefrom. The cleavage site between the signal peptide and the mature protein is located between position 30 and 31 of the amino acid sequence. The signal peptide-coding nucleic acid can be operatively linked to a protein-coding nucleic acid and can be used for the recombinant production of proteins in a process in which a transformed host cell is provided, the host cell is cultured under conditions which lead to an expression of the nucleic acid and to production and secretion of the polypeptide coded thereby and the resulting polypeptide is isolated from the culture medium. Prokaryotic organisms are preferably used as host cells in particular gram-positive organisms of the genus bacillus.

Surprisingly it was found that the recombinant production of S-layer proteins is not only possible in gram-positive prokaryotic host cells but also in gram-negative prokaryotic host cells. In this case the S-layer protein is not formed in the interior of the host cell in the form of ordered inclusion bodies but rather

unexpectedly in the form of ordered monomolecular layers.

Hence one subject matter of the present invention is a process for the recombinant production of S-layer proteins characterized in that (a) a gram-negative prokaryotic host cell is provided which is transformed with a nucleic acid coding for an S-layer protein selected from (i) a nucleic acid which comprises the nucleotide sequence shown in SEQ ID NO. 1 from position 1 to 3684 optionally without the section coding for the signal peptide, (ii) a nucleic acid which comprises a nucleotide sequence corresponding to the nucleic acid from (i) within the scope of the degeneracy of the genetic code and (iii) a nucleic acid which comprises a nucleotide sequence which hybridizes with the nucleic acids from (i) or/and (ii) under stringent conditions; (b) the host cell is cultured under conditions which lead to an expression of the nucleic acid and to production of the polypeptide coded thereby and (c) the resulting polypeptide is isolated from the host cell.

The term "stringent hybridization" is understood within the sense of the present invention to mean that a hybridization still also occurs after washing at 55°C, preferably 60°C in an aqueous low salt buffer (e.g. 0.2 x SSC) (see also Sambrook et al. (1989), Molecular Cloning. A Laboratory Manual).

The process according to the invention is carried out in gram-negative prokaryotic host cells. In this process an ordered S-layer protein structure is surprisingly obtained in the cell interior. Enterobacteria, in particular E. coli, are preferably used as host cells.

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The E. coli strain ~~pop2125~~ pop2136 which was deposited on the 31.01.1996 at the "Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH", Mascheroder Weg 1b, D 38124 Braunschweig under the file number DSM 10509 is particularly preferred.

The process according to the invention can also be used to isolate recombinant S-layer proteins. For this one uses a nucleic acid coding for the S-layer protein which contains one or several insertions which code for peptide or polypeptide sequences. These insertions can, on the one hand, only code for peptides with a few amino acids e.g. 1-25 amino acids. On the other hand, the insertions can also code for larger polypeptides of for example up to 1000 amino acids and preferably up to 500 amino acids without loss of the ability of the S-layer protein to form a correctly folded structure. In addition to the insertions the recombinant S-layer protein can also have amino acid substitutions, in particular substitutions of individual amino acids in the region of the insertion sites as well as optionally deletions of individual amino acids or short amino acid sections of up to 30 amino acids.

Regions between the positions 1-1200 and 2200-3000 of the nucleotide sequence shown in SEQ ID NO.1 are preferred as insertion sites for polypeptide-coding sequences. Particularly preferred insertion sites are the NruI cleavage site at position 582, the PvuII cleavage site at position 878, the SnaB-I cleavage site at position 917, the PvuII cleavage site at position 2504 and the PvuII cleavage site at position 2649. It was already possible to demonstrate the insertion of a nucleic acid coding for streptavidin into the NruI cleavage site at position 581.

The peptide or polypeptide-coding insertions are preferably selected from nucleotide sequences which code for cysteine residues, regions with several charged amino acids, e.g. Arg, Lys, Asp or Glu, or Tyr residues, DNA-binding epitopes, antigenic, allergenic or immunogenic epitopes, metal-binding epitopes, streptavidin, enzymes, cytokines or antibody-binding proteins.

A particularly preferred example of an insertion into the nucleic acid coding for the S-layer protein is a nucleotide sequence coding for streptavidin. In this manner it is possible to obtain universal carrier molecules which are suitable for coupling biotinylated reagents and for detection in immunological or hybridization test procedures.

A further preferred example of insertions are antigenic, allergenic or immunogenic epitopes e.g. epitopes from pathogenic microorganisms such as bacteria, fungi, parasites etc. and viruses, or epitopes from plants or epitopes against endogenous substances e.g. cytokines as well as against toxins in particular endotoxins. Particularly preferred examples of immunogenic epitopes are epitopes from herpes viruses such as the herpes virus 6 or pseudorabies virus (Lomniczi et al., J. Virol. 49 (1984), 970-979), in particular epitopes from the genes gB, gC or/and gD, or foot-and-mouth disease virus (FMDV), in particular epitopes from the gene sections which code for VP1, VP2 or/and VP3. The immunogenic epitopes can be selected such that they promote an antibody-mediated immune reaction or/and the production of a cellular immune reaction e.g. by stimulation of T cells. Examples of suitable allergenic epitopes are birch pollen allergens e.g. Bet v I (Ebner

et al., J. Immunol. 150 (1993) 1047-1054). Antigenic epitopes are additionally particularly preferred which are able to bind and filter out endogenous or exogenous substances such as cytokines or toxins from serum or other body fluids. Such epitopes can include components of cytokine or toxin receptors or of antibodies against cytokines or toxins.

On the other hand the insertions can also code for enzymes. Preferred examples are enzymes for the synthesis of polyhydroxybutyric acid e.g. PHB synthase. Incorporation of PHB synthase into the S-layer can lead to the formation of a molecular spinning nozzle under suitable conditions when the substrate hydroxybutyric acid is provided. A further preferred example of an enzyme is bacterial luciferase. In this case when the enzyme substrate, an aldehyde, is supplied and O_2 is present, a molecular laser can be obtained.

Insertions are likewise preferred which code for cytokines such as interleukins, interferones or tumour necrosis factors. These molecules can for example be used in combination with immunogenic epitopes to prepare vaccines.

Finally insertions are also preferred which code for antibody binding proteins such as protein A or protein G or for DNA-binding or/and metal-binding epitopes such as the leucine zipper, zinc finger etc.

Thus for the first time a cell is provided by the present invention which contains immobilized recombinant polypeptides in a native form e.g. active enzymes in the cytoplasm. In this manner 50,000 - 200,000 e.g. ca.

100,000 recombinant molecules can be immobilized per m^2 recombinant S-layer. Up to 3000 m^2 S-layer can be obtained per kg recombinant E. coli cells.

In the method according to the invention the nucleic acid coding for the S-layer protein is preferably used in operative linkage with a nucleic acid coding for a signal peptide of gram-positive bacteria i.e. the signal peptide-coding nucleic acid is located on the 5' side of the S-layer protein-coding nucleic acid. Surprisingly it was found that the presence of such signal peptide sequences, which are not cleaved in the gram-negative host cells used in the invention, can improve the stability of the S-layer structures. The nucleic acid coding for the signal peptide particularly preferably comprises (a) the signal peptide-coding section of the nucleotide sequence shown in SEQ ID NO. 1, (b) a nucleotide sequence corresponding to the sequence from (a) within the scope of the degeneracy of the genetic code or/and (c) a nucleotide sequence which is at least 80 % and in particular at least 90 % homologous to the sequences from (a) or/and (b).

Yet a further subject matter of the present invention is a nucleic acid which codes for a recombinant S-layer protein and is selected from (i) a nucleic acid which comprises the nucleotide sequence shown in SEQ ID NO.1 from position 1 to 3684 optionally without the signal peptide-coding section (ii) a nucleic acid which comprises a nucleotide sequence corresponding to a nucleic acid from (i) within the scope of the degeneracy of the genetic code and (iii) a nucleic acid which comprises a nucleotide sequence which hybridizes under stringent conditions with the nucleic acids from (i) or/and (ii).

The coding nucleotide sequence of the S-layer gene sbsA from B.stearothermophilus including the signal peptide-coding section is shown in SEQ ID NO. 1. The signal peptide-coding section extends from position 1 to 90 of the nucleotide sequence shown in SEQ ID NO. 1. The section coding for the mature SbsA polypeptide extends from position 91 to 3684.

The sbsA gene of B.stearothermophilus codes for a protein with a total of 1228 amino acids including an N-terminal signal peptide with 30 amino acids (SEQ ID NO. 2). The cleavage site between the signal peptide and the mature protein is located between position 30 and 31 of the amino acid sequence. The signal peptide has a basic amino-terminal domain followed by a hydrophobic domain.

Sequence comparisons with other signal peptides indicate a certain homology to signal peptides of extracellular proteins in bacilli such as alkaline phosphatase and neutral phosphatase of B.amyloliquefaciens (Vasantha et al., J. Bacteriol. 159 (1984), 811-819) as well as with the signal peptides for the B.sphaericus gene 125 (Bowditch et al., J. Bacteriol. 171 (1989), 4178-4188) and the OWP gene of B.brevis (Tsuboi et al., J. Bacteriol. 168 (1986), 365-373).

A further subject matter of the present invention is a recombinant vector which contains at least one copy of a nucleic acid according to the invention. The vector is preferably replicatable in prokaryotes. The vector is particularly preferably a prokaryotic plasmid.

Yet a further subject matter of the present invention is

a host cell which is transformed with a nucleic acid or a recombinant vector according to the present invention. The cell is preferably a gram-negative prokaryotic organism and most preferably an E. coli cell. The cell according to the invention can contain a recombinant S-layer structure in its interior. Methods for the transformation of cells with nucleic acids are general state of the art (cf. Sambrook et al., supra) and therefore do not need to be elucidated.

Yet a further subject matter of the present invention is a recombinant S-layer protein which contains at least one peptide insertion or/and polypeptide insertion within the amino acid sequence shown in SEQ ID NO. 2. Preferred examples of peptide insertions and polypeptide insertions have already been elucidated.

A recombinant S-layer structure can be assembled from recombinant S-layer protein molecules according to the invention which contain at least one recombinant S-layer protein according to the invention as a subunit. Furthermore it is preferred that the S-layer structure according to the invention also contains non-modified S-layer proteins as diluent molecules. The non-modified S-layer proteins are preferably present in a molar proportion of 10-99 % relative to the total S-layer proteins.

The S-layer structure according to the invention can comprise several layers that are covalently linked together or by means of affinity binding. Covalent linkages can for example be introduced by insertions of cysteine residues and a subsequent formation of cystine bridges. Linkages by affinity binding comprise for

example antibody-antigen, antibody-protein A or antibody-protein G or streptavidin-biotin interactions.

S-layer structures which contain recombinant S-layer proteins can optionally also be prepared in a carrier-bound form. For this the S-layer structure can be reassembled from individual units in the presence of a peptidoglycan carrier to for example produce peptido-glycan layers which are covered on one or on both sides with an S-layer structure. Another method of preparing carrier-bound S-layer structures is to produce an S-layer layer at an interface between two media e.g. water/air and to immobilize this layer on a solid phase e.g. a filter membrane (cf. e.g. Pum and Sleytr (1994), Thin Solid Films 244, 882-886; Küpcü et al., (1995), Biochim. Biophys. Acta 1235, 263-269).

The recombinant S-layer proteins and S-layer structures according to the invention are suitable for a multitude of applications. An application as a vaccine or adjuvant is particularly preferred in which case recombinant S-layer proteins are used which contain immunogenic epitopes of pathogens and/or endogenous immuno-stimulatory polypeptides such as cytokines. In this application it is not absolutely necessary to purify the recombinant S-layer proteins. Instead they can for example be used in combination with a bacterial ghost which optionally contains additional immunogenic epitopes in its membrane.

The preparation of suitable "bacterial ghosts" is described for example in the International Patent application PCT/EP91/00967 to which reference is herewith made. In this application modified bacteria are

disclosed which are obtainable by transformation of a gram-negative bacterium with the gene of a lytically active membrane protein from bacteriophages, with the gene of a lytically active toxin release protein or with genes which contain partial sequences thereof which code for lytic proteins, culturing the bacterium, expression of this lysis gene and isolation of the resulting bacterial ghost from the culture medium.

A recombinant protein, which is obtainable by expression of a recombinant DNA in these gram-negative bacteria, can be bound to the membrane of these bacteria as described in the European Patent 0 516 655. This recombinant DNA comprises a first DNA sequence which codes for a hydrophobic, non-lytically active membrane-integrating protein domain which has an α-helical structure and is composed of 14-20 amino acids which can be flanked N- and C-terminally by 2-30 arbitrary amino acids in each case. A second DNA sequence is in operative linkage with this first DNA sequence which codes for a desired recombinant protein. Furthermore the gram-negative bacterium contains a third DNA sequence which is under a different control from the first and second DNA sequences and codes for a lytically active membrane protein from bacteriophages or a lytically active toxin release protein or for their lytically active components. So-called "bacterial ghosts" are obtained by expression and lysis of such recombinant gram-negative bacteria which contain an intact surface structure with immunogenic epitopes bound to the surface.

When these bacterial ghosts are combined with recombinant S-layers according to the invention vaccines and adjuvants can be produced which have particularly

advantageous properties.

A further particularly preferred application for recombinant S-layer proteins and S-layer structures is their use as an enzyme reactor. Such an enzyme reactor can for example be formed by a cell which contains a recombinant S-layer structure according to the invention in its interior. On the other hand the enzyme reactor can also be formed from isolated and in vitro reassembled S-layer structures or combinations of various S-layer structures.

It was found that the gram-positive bacterium B.stearothermophilus PV72 has an additional S-layer protein in addition to SbsA which is subsequently denoted as SbsB (Sara and Sleytr (1994), J. Bacteriol. 176, 7182-7189). It was possible to isolate and characterize the sbsB gene by amplification using suitable nucleic acid primers. The coding nucleotide sequence of the S-layer gene sbsB from B.stearothermophilus including the signal peptide-coding section which extends from position 1 to 93 of the nucleic acid sequence is shown in SEQ ID NO.5. The amino acid sequence derived therefrom is shown in SEQ ID NO.6. The sbsB gene codes for a protein with a total of 921 amino acids including an N-terminal signal peptide with 31 amino acids.

One subject matter of the present invention is hence a nucleic acid which codes for an S-layer protein and is selected from

(i) a nucleic acid which comprises the nucleotide sequence from position 1 to 2763 shown in SEQ ID NO.5 optionally without the signal peptide-

coding section,

(ii) a nucleic acid which comprises a nucleotide sequence corresponding to the nucleic acid from (i) within the scope of the degeneracy of the genetic code and

(iii) a nucleic acid which comprises a nucleotide sequence that hybridizes with the nucleic acids from (i) or/and (ii) under stringent conditions.

As in the case of the sbsA gene, it is also possible to insert at least one nucleic acid insertion coding for a peptide or polypeptide into the sbsB gene within the region coding for the S-layer protein. With regard to preferred examples of insertions in the sbsB gene reference is made to the previous statements regarding the sbsA gene.

Yet a further subject matter of the present invention is a vector which contains at least one copy of an sbsB gene optionally containing an insertion. This vector can be replicated in eukaryotes, prokaryotes or in eukaryotes and prokaryotes. It can be a vector that can be integrated into the genome of the host cell or a vector which is present extrachromosomally. The vector according to the invention is preferably a plasmid in particular a prokaryotic plasmid.

Yet a further subject matter of the present invention is a host cell which is transformed with an sbsB gene wherein the sbsB gene optionally can contain an insertion. The host cell can be a eukaryotic as well as a prokaryotic cell. The cell is preferably a prokaryotic organism. Gram-positive organisms e.g. organisms of the genus bacillus as well as gram-negative organisms such

as enterobacteria in particular E. coli are preferred. Methods for transforming eukaryotic and prokaryotic cells with nucleic acids are known and therefore do not need to be elucidated in detail.

The present invention also concerns an SbsB protein i.e. an S-layer protein which is coded by a nucleic acid as defined above. Recombinant SbsB proteins are particularly preferred which contain one or several peptide or/and polypeptide insertions within the sbsB sequence. The SbsB part of a polypeptide according to the invention particularly preferably has a homology of at least 80 % and in particular of at least 90 % to the amino acid sequence shown in SEQ ID NO.6.

A recombinant S-layer structure can also be assembled from the recombinant SbsB-S-layer protein molecules analogous to the recombinant SbsA-S-layer structure. In this structure the non-modified S-layer proteins are preferably present in a molar proportion of 10-99 % relative to the total S-layer proteins.

The applications for the recombinant SbsB-S-layer proteins and S-layer structures according to the invention also correspond to the applications for SbsA mentioned above. In this connection its use as a vaccine or adjuvant or as an enzyme reactor is noteworthy.

Recombinant S-layer proteins are obtainable by a process in which

(a) a host cell is provided which contains a nucleic acid coding for an S-layer protein which contains a peptide-coding or polypeptide-coding insertion within the region coding for the S-layer protein,

(b) the host cell is cultured under conditions which lead to an expression of the nucleic acid and to production of the polypeptide coded by it and
(c) the resulting polypeptide is isolated from the host cell or from the culture medium.

In a first preferred embodiment of this process a recombinant SbsA-S-layer protein is prepared i.e. the nucleic acid coding for the recombinant S-layer protein is selected from

(i) a nucleic acid which comprises the nucleotide sequence from position 1 to 3684 shown in SEQ ID NO.1 optionally without the signal peptide-coding section,
(ii) a nucleic acid which comprises a nucleotide sequence corresponding to the nucleic acid from (i) within the scope of the degeneracy of the genetic code and
(iii) a nucleic acid which comprises a nucleotide sequence which hybridizes with the nucleic acids from (i) or/and (ii) under stringent conditions.

In a second preferred embodiment a recombinant SbsB-S-layer protein is prepared i.e. the nucleic acid coding for the recombinant S-layer protein is selected from

(i) a nucleic acid which comprises the nucleotide sequence from position 1 to 2763 shown in SEQ ID NO.5 optionally without the signal peptide-coding section,
(ii) a nucleic acid which comprises a nucleotide sequence corresponding to the nucleic acid from (i) within the scope of the degeneracy of the genetic code and
(iii) a nucleic acid which comprises a nucleotide sequence which hybridizes with the nucleic acids

from (i) or/and (ii) under stringent conditions.

In addition to the recombinant SbsA and SbsB-S-layer proteins from B.stearothermophilus it is, however, also possible to prepare recombinant S-layer proteins from other organisms (cf. e.g. Peyret et al., (1993), supra).

The recombinant S-layer proteins can on the one hand be produced in a heterologous host cell i.e. in a host cell which originally contains no S-layer gene. Examples of such heterologous host cells are gram-negative prokaryotic organisms such as E. coli.

However, the heterologous expression of S-layer proteins can also take place in gram-positive prokaryotic organisms such as B. subtilis. For this integration vectors are preferably used which contain a native or/and a recombinant S-layer gene. When the native signal sequences are used the S-layer proteins are secreted into the culture supernatant.

However, it is often preferable to produce the recombinant S-layer proteins in homologous host cells i.e. host cells which originally contain a natural S-layer gene. In one embodiment of this homologous expression the recombinant S-layer gene is introduced into the host cell in such a way that the host cell is still able to express a further S-layer gene which codes for a non-modified S-layer protein. The non-modified S-layer protein is preferably capable of forming an S-layer structure that is compatible with the recombinant S-layer protein. An example of this embodiment of homologous expression is a B.stearothermophilus PV72 cell which contains intact natural sbsA genes or/and

sbsB genes and is transformed with a plasmid which contains a recombinant S-layer gene.

In a second embodiment the homologous expression can occur in a host cell in which the intact S-layer gene originally present has been inactivated. Consequently in this embodiment no further S-layer gene is expressed in the host cell which codes for a non-modified S-layer protein which is able to form a compatible S-layer structure with the recombinant S-layer protein. A specific example of such a host cell is a B.stearothermophilus PV72 cell in the genome of which a gene coding for a recombinant S-layer protein has been introduced, e.g. by homologous recombination, which replaces the original S-layer gene. A further example of such a host cell is a B.stearothermophilus cell in which the native S-layer gene has been inactivated e.g. by site-specific mutagenesis or/and homologous recombination and is transformed with a vector containing a recombinant S-layer gene.

Gram-positive prokaryotic organisms are usually used as host cells for the homologous expression of recombinant S-layer genes. B.stearothermophilus PV72 is particularly preferred as a host cell which can be cultured at a high temperature in a defined synthetic medium (Schuster et al., (1995), Biotechnol. and Bioeng. 48: 66-77).

The present invention is further elucidated by the following examples and figures.

SEQ ID NO.1 shows the complete nucleotide sequence of the coding section of the S-layer gene sbsA of B.stearothermophilus;

SEQ ID NO.2 shows the amino acid sequence derived therefrom;

SEQ ID NO.3 shows the nucleotide sequence of the primer T5-X;

SEQ ID NO.4 shows the nucleotide sequence of the primer E;

SEQ ID NO.5 shows the complete nucleotide sequence of the coding section of the S-layer gene sbsB of B.stearothermophilus;

SEQ ID NO.6 shows the amino acid sequence derived therefrom;

SEQ ID NO.7 shows the nucleotide sequence of a partial fragment of the streptavidin gene;

SEQ ID NO.8 shows the nucleotide sequence of the primer NIS 2AG;

SEQ ID NO.9 shows the nucleotide sequence of the primer LIS C3;

Fig. 1 shows a schematic representation of the sbsA PCR fragment used to prepare the recombinant vector pBK4;

Fig. 2 shows a schematic representation of peptide insertions in the amino acid sequence of the SbsA S-layer protein and

Fig. 3 shows a schematic representation of amino acid substitutions and amino acid insertions in recombinant S-layer proteins.

EXAMPLES:

1. Bacterial strains, media and plasmids

Gram-positive bacteria of the strain Bacillus stearothermophilus PV72 were cultured at 58°C in SVIII medium (Bartelmus and Perschak, Z.Zuckerrind. 7 (1957), 276-281). Bacteria of the strain E. coli pop2135 (endA, thi,

hsdR, malT, cI857, λpR, malPQ) were cultured in LB medium (Sambrook et al., (1989), supra). Ampicillin was added to the medium at a final concentration of 100 μg/ml to select for transformants. The plasmid pPLcAT10 (λpL, bla, colE1) (Stanssens et al., Gene 36 (1985), 211-223) was used as the cloning vector.

2. Manipulation of DNA fragments

Restriction analysis of DNA, agarose gel electrophoresis and cloning of DNA fragments were carried out according to the standard methods described in Sambrook et al. (1989), supra.

Competent cells were transformed by electroporation using a Bio-Rad gene pulser (Bio-Rad Laboratories, Richmond, Calif. USA) according to the manufacturer's instructions.

Plasmid DNA was isolated by the method of Birnboim and Doly (Nucleic Acids Res. 7 (1979), 1513-1523). Chromosomal DNA was isolated according to the method described in Ausubel et al. (Current Protocols in Molecular Biology (1987), New York, John Wiley).

Restriction endonucleases and other enzymes were obtained from Boehringer Mannheim, New England Biolabs or Stratagene and used according to the manufacturer's instructions.

3. DNA sequencing

The DNA sequences of the 5' regions and the 3' regions

(including the region coding for the signal sequence) of the gene sbsA in the vector pPLcAT10 were determined by the dideoxy chain termination method of Sanger et al. The primers used for sequencing were constructed on the basis of the already published sbsA sequence (Kuen et al. Gene 145 (1994), 115-120).

4. PCR amplification of sbsA

The PCR amplification of the sbsA gene was carried out in a reaction volume of 100 µl in which 200 µM deoxynucleotides, 1 U Pfu-polymerase (Stratagene), 1 x Pfu-reaction buffer, 0.5 µM of each oligonucleotide primer and 100 ng genomic DNA from B.stearothermophilus as a template were present. The amplification was carried out for 30 cycles in a thermocycler (Biomed thermocycler 60). Each cycle was composed of a denaturing step of 1.5 min at 95°C, an annealing step of 1 min at 56°C and 1 min at 50°C as well as an extension step of 2 min at 72°C.

The primer T5-X shown in the sequence protocol as SEQ ID NO.3 which flanks the 5' region of sbsA and contains an XbaI site and the primer E shown in the sequence protocol in SEQ ID NO.4 which flanks the 20 nucleotide upstream region of the transcription terminator of the sbsA sequence and contains a BamHI site were used as primers.

The products amplified by PCR were electrophoretically separated on a 0.8 % agarose gel and purified for cloning using the system from Gene Clean (BIO101 La Jolla, Calif. USA).

5. Cloning of the sbsA gene into the vector pPLcAT10

The sbsA gene obtained by PCR with a length of 3.79 kb was purified and cleaved with the restriction endonucleases XbaI and BamHI. The resulting XbaI-BamHI fragment was cloned into the corresponding restriction sites of the vector pPLcAT10 so that the sbsA gene was under transcriptional control of the pL promoter located upstream. The ATG start codon of the sbsA sequence was reconstructed by the cloning procedure. The cloned sbsA sequence contained the N-terminal signal sequence of sbsA and ended 20 nt after the transcription terminator. After ligation of the vector DNA with the sbsA fragment, the E. coli strain pop2135 was transformed by electro-transformation. The resulting clones were subjected to a DNA restriction analysis. A positive clone was sequenced in order to verify the correct sequence transitions at the 5' and 3' ends. This clone was named pBK4.

A schematic representation of the 3.79 kb XbaI sbsA fragment and its location in the multiple cloning site of the plasmid pBK4 is shown in Fig. 1 (abbreviations: tT: transcription terminator; ori: origin of the DNA replication; amp: ampicillin resistance gene).

6. Recombinant expression of the sbsA gene in E. coli

E. coli pop2135/pBK4 cells were cultured at 28°C until an optical density OD_{600} of 0.3 was reached. Then the expression of sbsA was induced by increasing the culture temperature from 28°C to 42°C. 1.5 ml aliquots were taken before and 1, 2, 3 and 5 hours after induction of the sbsA expression. E. coli pop2135/pPLcAT10 (cultured under the same conditions) and B.stearothermophilus PV72

were used as controls.

Culture supernatants and cell extracts from all samples were examined for the expression of S-layer proteins by SDS-PAGE and Western immunoblotting.

An additional strong protein band with the same molecular weight as the wild type SbsA protein was found in extracts from E. coli cells transformed with pBK4. No degradation products of SbsA itself were found in a period of up to 5 hours after induction of expression. Thus presumably the S-layer protein sbsA is stable in E. coli and is not degraded by proteases.

A densitometric determination of the relative amount of SbsA protein was carried out. At a time point of 4 hours after induction the sbsA protein was in a proportion of ca. 16 % relative to the total cellular protein.

The SbsA protein produced in E. coli migrated in the SDS gel slightly more slowly than the natural SbsA protein from B.stearothermophilus. Experiments to determine the N-terminal amino acid sequence of the SbsA protein by Edman degradation were not successful due to a blocking of the N-terminus. Thus presumably the signal sequence was not cleaved in E. coli.

A Western blot analysis of total cell extracts and culture supernatants of E. coli/pBK4 also only yielded a single sbsA-specific protein band with a slightly higher molecular weight than wild type SbsA protein from stearothermophilus.

For the Western blot the proteins were transferred onto a nitrocellulose membrane and incubated with a polyclonal antiserum against SbsA from rabbits. The preparation of this antiserum is described in Egelseer et al. (J. Bacteriol. 177 (1995), 1444-1451). A conjugate of goat anti-rabbit IgG and alkaline phosphatase was used to detect bound SbsA-specific antibodies.

No SbsA protein could be detected from supernatants from E. coli cells transformed with pBK4 even after induction of sbsA gene expression. This shows that SbsA is not exported into the surrounding medium.

7. Location and organisation of the S-layer protein SbsA in the cytoplasm of E. coli

Cells of E. coli pop2135/pBK4 which were harvested from cultures 1, 2, 3 and 5 hours after induction of the S-layer protein expression were examined for the intracellular organisation of sbsA. Non-induced cells cultured at 28°C and cells of B.stearothermophilus PV72 were examined as controls.

For this whole cells of both organisms were fixed and embedded in detection resin according to the method of Messner et al. (Int. J.Syst.Bacteriol. 34 (1984), 202-210). Subsequently ultrathin sections of the embedded preparations were prepared and stained with uranyl acetate.

The cytoplasm of non-induced E. coli cells exhibited the typical granular structure which did not change even when the OD of the suspensions increased. Longitudinal

sections of E. coli cells which were harvested 1 hour after induction of the S-layer protein expression exhibited parallel, leaf-like structures in the cytoplasm. From cross sections it was apparent that these structures have a concentric arrangement.

The amount of leaf-like structures considerably increased between 1 and 2 hours after induction of the sbsA expression and afterwards remained essentially constant.

The sbsA protein recombinantly produced in E. coli could also be detected by immunogold labelling with sbsA-specific antibodies. An ordered structure of the recombinantly produced SbsA protein was also found with this detection method.

It was clearly apparent from these morphological data that the SbsA protein did not aggregate to form irregular inclusion bodies but rather formed monomolecular S-layer crystals. A remarkable property of the SbsA-S-layer layers assembled in E. coli was the concentric arrangement at defined distances. The presence of the signal sequence did not interfere with correct assembly.

8. Preparation of recombinant sbsA-S-layer genes

8.1 Insertion of a 6 bp long DNA sequence

A modified kanamycin cassette (1.3 kb) was used for the site-specific insertion mutagenesis of the sbsA gene which was isolated by cleavage of the plasmid pWJC3

(obtained from W.T. McAllister, New York) by SmaI. The cassette was ligated into five different blunt-ended restriction sites of the sbsA gene, i.e. into the NruI site at position bp 582 (pSL582), into the SnaBI site at position bp 917 (pSL917) and into each of the PvuII sites at positions bp 878 (pSL878), bp 2504 (pSL2504) and bp 2649 (pSL2649). After selection of kanamycin-resistant clones, the cassette was removed from the insertion site by cleavage with ApaI followed by a religation of the S-layer plasmid pBK4. The cutting out and religation procedure left an insertion of 6 bp CCCGGG (ApaI restriction site). The system of this linker insertion is shown schematically in Fig. 2.

The resulting recombinant S-layer genes code for modified sbsA proteins elongated by 2 amino acids.

The specific changes in the primary structure of the sbsA proteins are shown in Fig. 3. In the clone pSL582 the insertion led to the incorporation of glycine and proline between the amino acids 194 and 195 at the N-terminus of the SbsA protein. The amino acids alanine and arginine were inserted in the clone pSL917 between the amino acids 306 and 307. In the clone pSL2649 glycine and proline were inserted between the amino acids at positions 883 and 884. An insertion of alanine and proline between the amino acids 293 and 294 was obtained in the clone pSL878. Furthermore the alanine at position 293 was substituted by glycine. In the clone pSL2504 the amino acids alanine and proline were inserted between the amino acids 835 and 836 and the alanine at position 835 was replaced by glycine.

All clones obtained by insertion mutagenesis retained

their ability to synthesise the S-layer protein.

In order to test the ability of the modified proteins to assemble into S-layer structures, ultrathin longitudinal sections of whole cells which had been cultured for 4 hours under inductive conditions were prepared according to the procedure described in section 7. It was found that the cytoplasm of all five clones is filled with parallel, leaf-like structures which follow the curve of the cell poles. There were no morphological differences of the cytoplasm in the 5 different clones examined. Exactly the same leaf-like structures were found as in the assembly of the wild type SbsA protein in E. coli (section 7).

8.2 Insertion of a DNA sequence coding for streptavidin

In order to examine whether the insertion of larger protein sequences into the SbsA protein can also be tolerated, a DNA fragment coding for a part of streptavidin (160 amino acids) provided with ApaI linkers (SEQ ID NO.7) was gene inserted into the ApaI restriction site of the sbsA clones pSL582, pSL878, pSL917 and pSL2649 prepared in the example on page 1. The streptavidin sequence was inserted in SL582 in the codon 197, in pSL878 between codon 295 and 296, in pSL917 in the codon 308 and 309 and in pSL2649 in the codon 886. It was possible to detect the expression of SbsA-streptavidin fusion proteins in all constructs by SDS-PAGE and immunoblots. It was found by EM analysis that a self assembly of the S-layer structure was possible in the fusion proteins containing insertions in the codon 197 and between the codons 295 and 296.

The SbsA-streptavidin fusion proteins can be isolated as monomers and reassembled to form homogeneous SbsA-streptavidin S-layers or mixed SbsA-streptavidin/SbsA-S-layers. They can be used to bind biotinylated substances as well as to determine the binding capacity of enzymes and other bound molecules.

8.3 Insertion of a DNA sequence coding for BetvI

A DNA sequence coding for the open reading frame of BetvI (161 amino acids) the main pollen allergen of the birch (Ferreira et al., J. Biol. Chem. 268 (1993), 19574-19580) was inserted at the ApaI site into the sbsA clone pSL878. It was possible to detect the expression of an SbsA-BetvI fusion protein which contained an immunologically active BetvI domain.

The resulting fusion protein can be used for therapeutic or diagnostic purposes. Hence it can be attempted by administration of the fusion protein to convert a T_H2-directed IgE antibody reaction into a T_H1-mediated reaction against BetvI. In this manner it is possible to suppress the occurrence of symptoms of a pollen allergy. Furthermore SbsA-BetvI fusion proteins can be used to test for anti-BetvI antibody concentrations or/and to reduce high concentrations of anti-BetvI IgE.

8.4 Insertion of a DNA sequence coding for a pseudorabies virus antigen

The DNA sequence coding for the gB epitope SmaBB (255 amino acids) (nucleotides 489-1224 corresponding to the coordinates according to the EMBL-Seq: HEHSSGP2) from the pseudorabies virus was inserted into SSpI site of

the sbsA gene after nt 3484 (between codon 1161 and 1162). It was possible to detect the expression of SbsA-SmaBB fusion proteins.

The fusion proteins can be used to test gB-specific immune reactions. A Western blot analysis using a monoclonal antibody which corresponds to the inserted sequence showed the immunological activity of the viral domain within the recombinant SbsA-SmaBB proteins.

8.5 Insertion of a DNA sequence coding for the PHB synthase (PhbC) from Alcaligenes eutrophus H16

A regular arrangement of polypeptide structures with enzymatic activity on the surface of S-layers is an important goal in the production of immobilized enzymes within a living cell and in the case of the 590 amino acid long PHB synthase for the production of a molecular machine for biopolymer synthesis.

The phbC gene was isolated by PCR from the plasmid p4A (Janes et al., Molecular characterisation of the poly-β-hydroxy-butyrate biosynthesis in Alcaligenes eutrophus H16. In: Novel Biodegradable Microbial Polymers (publisher Daves, E.A.), pp 175-190 (1990), Kluver, Dordrecht) as a 1770 nt long DNA fragment (corresponding to an open reading frame of 590 amino acids) and inserted into the ApaI cleavage site of the sbsA clone pSL878 to obtain the plasmid pSbsA-PhbC. It was possible to detect the expression of an SbsA-PhbC fusion protein of ca. 195 kD in an E. coli cell transformed with this plasmid. When two copies of the phbC gene were inserted one behind the other into the ApaI site of pSL878, it was possible to detect the expression of a fusion

protein of ca. 260 kD.

For a functional test of the enzymatic activity of the SbsA-PhbC construct, the E. coli cells which contained the plasmid pSbsA-PhbC were co-transformed with the plasmid pUMS which contains the β-ketothiolase (PhbA) and the acetoacetyl-CoA reductase (PhbB) from A. eutrophus (Kalousek et al., Genetic engineering of PHB-synthase from Alcaligenes eutrophus H16. In: Proceedings of the International Symposium on Bacterial Polyhydroxy-alkanoates, pp 426-427 (1993), publisher Schlegel H. G., Steinbüchel A. Goltze Press, Göttingen). The poly-β-hydroxybutyrate formation in the co-transformed E. coli cells was detectable by staining with Sudan black, gas chromatography and electron microscopy. These findings show that the SbsA-PhbC construct is enzymatically active and represents a successful example of the immobilization of enzymes on intracellular S-layer matrices.

8.6 Insertion of a DNA sequence coding for a bacterial luciferase gene

A monocistronic LuxAB gene with a length of 2,070 nt which contains the fusion protein LuxAB composed of the two subunits LuxB and LuxB of the bacterial luciferase from Vibrio harveyi was isolated from the plasmid pT7-mut3 (Boylan et al., J. Biol. Chem. 264 (1989), 1915-1918) by PCR and inserted into the ApaI site of the clone pSL878 prepared in example 8.1 to obtain the plasmid pBK878-LuxAB. It was possible to detect the expression of an SbsA-PhbC fusion protein of ca. 207 kD in an E. coli cell transformed with this plasmid. The enzymatic activity of the fusion protein was

demonstrated by the method described in Boylan et al., Supra.

9. Isolation and characterization of the sbsB gene

The basis for the isolation of the sbsB gene was the amino acid sequence of the N-terminus as well as the sequence of three internal peptides of the SbsB protein. Starting with these peptide sequences, degenerate oligonucleotide primers were constructed and used for the PCR. In this manner a 1076 bp long PCR fragment from the chromosomal DNA of B.stearothermophilus was amplified, cloned and sequenced (corresponding to position 100-1176 of the sequence shown in SEQ ID NO.5).

The method of inverse PCR was used to amplify the sections on the 5' side and 3' side of the sbsB gene and stepwise overlapping DNA fragments were obtained with the aid of various primer combinations and sequenced.

The primer NIS 2AG shown in the sequence protocol as SEQ ID NO.8 which contains the 5' region of sbsB as well as the primer LIS C3 shown in the sequence protocol of SEQ ID NO.9 which contains the 3' region of sbsB were used as primers to amplify the complete sbsB gene.

The PCR fragment obtained in this manner which contains the nucleotide sequence shown in SEQ ID NO.5 with 5' and 3' BamHI restriction cleavage sites was cloned as described in example 5 into the vector pPLcAT10 in which the expression takes place under the control of the lambda PL promoter.

Furthermore the sbsB-PCR fragment with the 5' side EcoRI and 3' side BamHi cleavage site were cloned into the vector pUC18 in which the expression took place under the control of the lac promoter.

The detection of the sbsB expression was carried out as described in examples 6 and 7 by SDS gel electrophoresis and electron microscopy.

10. Preparation of recombinant sbsB-S-layer genes

Recombinant sbsB genes were prepared analogously to the methods described in example 8.

Thus in accordance with the method described in example 8.1, a 6 nt long DNA sequence containing an ApaI restriction cleavage site was introduced at various positions into the sbsB-layer gene. The recombinant sbsB clones pAK407, pAK481 and pAK1582 with ApaI cleavage sites at nt 407 (codon 136), 481 (codon 161/162) and 1582 (codon 528/529) were obtained in this manner. These clones obtained by insertion mutagenesis retained their ability to synthesize the S-layer protein and form S-layer structures.

Analogously to the method described in example 8.2, a DNA fragment coding for streptavidin was inserted into the ApaI restriction sites of the sbsB clones pAK407 and pAK481.

Analogously to example 8.4, a DNA sequence coding for the gB epitope SmaBB was inserted into the ApaI cleavage sites of the sbsB clones pAK481 and pAK1582. It was

possible to detect the expression of sbsB-SmaB fusion proteins of ca. 130 kD in the E. coli cells transformed with the resulting recombinant plasmids. When two copies of the SmaBB epitopes were inserted one behind the other into the ApaI cleavage site of pAK481 it was possible to detect the expression of a fusion protein of ca. 157 kD. The SmaBB domains of the fusion proteins were recognized by specific antibodies.

Analogously to example 8.6 it was possible to detect the expression of a 175 kD SbsB-LuxAB fusion protein when the LuxAB sequence was inserted into the ApaI cleavage site of pAK407.

11. Heterologous expression of sbsA and sbsB in Bacillus subtilis

The integration vector pX (Kim, L., Mogk, A. and Schumann W., Gene 181 (1996), 71-76: A xylose-inducible Bacillus subtilis integration vector and its application) was used for the heterologous expression of sbsA and sbsB in B. subtilis. The S-layer genes in the resulting recombinant expression vectors are under the transcriptional control of the xyl promoter. Transformants of B.subtilis containing an S-layer gene integrated in the chromosome exhibited an expression of large amounts of S-layer proteins in the supernatant of the cells which was inducible by addition of xylose to the growth medium. This shows that the signal sequences of sbsA and sbsB are recognized by the B. subtilis cell.

In an analogous manner it was possible to achieve a heterologous expression of recombinant sbsA and sbsB layer genes in B. subtilis.